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# Perioperative platelet and monocyte activation in patients with critical limb ischemia

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**Background:** Patients with critical limb ischemia (CLI) have a high rate of adverse cardiovascular events, particularly when undergoing surgery. We sought to determine the effect of surgery and vascular disease on platelet and monocyte activation in vivo in patients with CLI.

**Methods:** An observational, cross-sectional study was performed at a tertiary referral hospital in the southeast of Scotland. Platelet and monocyte activation were measured in whole blood in patients with CLI scheduled for infrainguinal bypass and compared with matched healthy controls, patients with chronic intermittent claudication, patients with acute myocardial infarction, and those undergoing arthroplasty (n = 30 per group). Platelet and monocyte activation were quantified using flow cytometric assessment of platelet-monocyte aggregation, platelet P-selectin expression, platelet-derived microparticles, and monocyte CD40 and CD11b expression.

**Results:** Compared with those with intermittent claudication, subjects with CLI had increased platelet-monocyte aggregates ( $41.7\% \pm 12.2\%$  vs  $32.6\% \pm 8.5\%$ , respectively), platelet microparticles ( $178.7 \pm 106.9$  vs  $116.9 \pm 53.4$ ), and monocyte CD40 expression ( $70.0\% \pm 12.2\%$  vs  $52.4\% \pm 15.2\%$ ;  $P < .001$  for all). Indeed, these levels were equivalent (P-selectin,  $4.4\% \pm 2.0\%$  vs  $4.9\% \pm 2.2\%$ ;  $P > .05$ ) or higher (platelet-monocyte aggregation,  $41.7\% \pm 12.2\%$  vs  $33.6\% \pm 7.0\%$ ;  $P < .05$ ; platelet microparticles,  $178.7 \pm 106.9$  vs  $114.4 \pm 55.0/\mu\text{L}$ ;  $P < .05$ ) than in patients with acute myocardial infarction. All platelet and monocyte activation markers remained elevated throughout the perioperative period in patients with CLI ( $P < .01$ ) but not those undergoing arthroplasty.

**Conclusions:** Patients undergoing surgery for CLI have the highest level of in vivo platelet and monocyte activation, and these persist throughout the perioperative period. Additional antiplatelet therapy may be of benefit in protecting vascular patients with more severe disease during this period of increased risk. (J Vasc Surg 2010;52:697-703.)

**Clinical Relevance:** Peripheral arterial disease is increasingly common and is associated with a significant risk of cardiovascular complications, especially at the time of surgery. Despite this, patients are poorly provided with evidence-based therapies such as antiplatelet and lipid-lowering medications. Platelets play a key role in the pathogenesis of atherothrombosis, with elevated levels of in vivo platelet activation prognostic of adverse clinical events. This study demonstrates, for the first time to our knowledge, significantly greater levels of platelet activation in patients with severe peripheral arterial disease compared with patients with acute myocardial infarction or patients undergoing other moderate- to high-risk surgical procedures. This further emphasizes the need for improved risk stratification and cardioprotection of this vulnerable group.

Patients with peripheral arterial disease (PAD) are at increased risk of adverse cardiovascular events.<sup>1</sup> This risk increases with the severity of disease, with progressive reductions in the ankle brachial pressure index (ABPI) being an independent predictor of cardiovascular outcome.<sup>2,3</sup> Cardiovascular

risk factor management is therefore the first-line treatment of patients with PAD. These patients are particularly at risk during the perioperative period. Myocardial infarction (MI) is the commonest life-threatening complication of major vascular surgery, with a reported perioperative incidence of 8% to 40%, depending on the diagnostic criteria.<sup>4-6</sup> This is comparable to the 30-day death and reinfarction rate of 8% to 20% seen in patients with acute coronary syndromes.<sup>7</sup> Despite this, patients with PAD often fail to receive evidence-based medical therapies. There is clearly an unmet need for improved risk stratification of these patients with appropriately tailored intensive medical regimens.

Atherogenesis and its complications are complex processes involving inflammatory and thrombotic mechanisms.<sup>8-10</sup> Monocyte adhesion to a damaged endothelium is a central step in the initiation and progression of atherosclerosis and appears to play a key role in plaque destabilization. In contrast, platelets can adhere to atherosclerotic lesions in the presence or absence of an overlying endothelium<sup>8</sup> and contribute to thrombus formation that is responsible for acute cardiovascular events. Hence, platelet and monocyte activation both predispose to plaque growth, instability, and rupture.

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Competition of interest: none.

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**Table I.** Inclusion and exclusion criteria

	<i>Healthy volunteers</i>	<i>Claudicants</i>	<i>Critical limb ischemia</i>	<i>Non-ST elevation MI</i>	<i>Arthroplasty</i>
Inclusion criteria	Age >50 y	Age >50 y Intermittent claudication	Age >50 y Rest pain and/or skin ulceration or necrosis	Age >50 y Cardiac chest pain plus ECG changes plus plasma troponin I > 0.2 µg/L	Age >50 y
		ABPI <1 and >0.2 Rutherford- Baker II, III	ABPI ≤ 0.2 Rutherford-Baker IV-VI		
Exclusion criteria	PAD  IHD, CVD Smoking Hypertension Diabetes Lipid-lowering agents Antiplatelet therapy  Antihypertensive agents Warfarin	Dual antiplatelet therapy  Warfarin	Dual antiplatelet therapy Warfarin	PAD	PAD  IHD, CVD Smoking Hypertension Diabetes Lipid-lowering agents Antiplatelet therapy Antihypertensive agents Warfarin

ABPI, Ankle-brachial pressure index; CVD, cerebrovascular disease; ECG, electrocardiogram; IHD, ischemic heart disease; MI, myocardial infarction.

Platelets and monocytes also directly interact to form platelet-monocyte aggregates that promote expression of vascular cell adhesion molecules and increase leucocyte adhesion to the inflamed endothelium.<sup>11</sup> In addition, the CD40/CD40 ligand dyad, which is expressed on the surface of activated monocytes and platelets, is a major inflammatory trigger that promotes release of inflammatory cytokines, adhesion molecules, and procoagulant activity.<sup>12</sup> Disruption of CD40/CD40L or platelet-monocyte aggregation leads to the retardation of atherosclerotic lesions in animal studies.<sup>13,14</sup>

Raised levels of platelet-monocyte aggregates and monocyte CD40 have been detected in smokers, patients with diabetes mellitus, acute coronary syndromes, and in those at risk of rethrombosis after percutaneous coronary interventions.<sup>15-18</sup> They are surrogate markers of clinical risk and are predictive of adverse cardiac events. Several studies have assessed platelet activation in peripheral atherosclerosis and demonstrated a progressive increase in activation with increasing severity of disease.<sup>19-21</sup> However, there have been relatively few reports of platelet activation and cellular inflammation at the time of operation,<sup>22-24</sup> and none, to our knowledge, have compared PAD patients with other high-risk populations.

We wished to investigate platelet and monocyte activation in patients with PAD specifically at the time of surgery and compare this to levels of activation in other high-risk populations. The high incidence of perioperative adverse cardiovascular events in patients with severe PAD could be mediated by increased systemic inflammation and platelet activation. These patients may potentially benefit from risk

stratification and appropriately tailored medical regimens, as occurs in patients with coronary atherosclerotic disease. This exploratory study may support the conduct of interventional projects aimed at reducing surrogate markers of clinical risk, before clinical trials of intensive antiplatelet and anti-inflammatory strategies.

## METHODS

This observational, cross-sectional study was performed with the approval of the local ethics committee and in accordance with the Declaration of Helsinki. All participants provided written informed consent.

**Participants.** Participants were recruited from five groups (n = 30 per group): (1) patients with nonthromboembolic CLI, (2) patients with chronic intermittent claudication, (3) patients with a non-ST segment elevation MI, (4) otherwise healthy patients undergoing hip or knee arthroplasty, and (5) healthy volunteers. The inclusion and exclusion criteria are summarized in Table I.

Patients with CLI, defined by the presence of rest pain or skin ulceration, or both, and an ABPI ≤0.2, who were scheduled to undergo infrainguinal bypass or amputation were recruited from the surgical vascular unit. Patients who had symptoms of intermittent claudication with an ABPI of <1 and >0.2, were recruited from the outpatient claudication clinic.

The 60 PAD patients received maintenance aspirin (75 mg daily) and statin therapy for at least 6 weeks before inclusion. We wished to examine platelet activation under the standard medical regimen; therefore, patients receiving clopidogrel or warfarin were excluded. At present, the only

evidence-based indication for dual antiplatelet therapy in PAD is coexisting history of recent (<6 months) coronary artery stenting or stroke.

Patients presenting to the coronary care unit and diagnosed with a non-ST elevation MI, defined as chest pain with electrocardiographic (ECG) changes and elevated plasma troponin I concentration ( $>0.2 \mu\text{g/L}$ ), were recruited.

We wished to examine the contribution of surgical stress (without underlying PAD) to in vivo platelet activation. After discussion with a panel of consultant vascular surgeons and anesthetists, we concluded knee or hip arthroplasty represented surgery of a similar magnitude to peripheral bypass or amputation and was likely to be performed in patients of a comparable age. Patients aged  $>50$  years undergoing elective arthroplasty were approached. To limit the effect of atherosclerosis on platelet activation, we excluded patients with a history of diabetes, hypertension, ischemic heart disease or stroke, smoking, or antiplatelet, antihypertensive, or statin use. Patients undergoing arthroplasty did not receive perioperative heparin according to the unit policy.

Sequential healthy individuals aged  $>50$  were recruited.

**Blood sampling.** Single baseline samples were taken from all 150 participants. In patients with non-ST segment elevation MI, samples were taken  $\leq 24$  hours of hospitalization and after the initiation of dual antiplatelet medication with aspirin and clopidogrel. To compare perioperative platelet and monocyte activation, three blood samples were taken from patients undergoing vascular or orthopedic surgery preoperatively, immediately postoperatively, and on the day after the operation.

Blood was drawn by venipuncture of a large antecubital vein using a 19-gauge needle. Care was taken to ensure a smooth blood draw without venous stasis. Samples were processed immediately. Blood for assessment of platelet-monocyte aggregates, platelet expression of P-selectin, and monocyte CD40 and CD11b, was collected into tubes containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences, Cambridge, UK). Blood for the assessment of platelet microparticles was collected into sodium citrate. Platelet-poor plasma was prepared by centrifugation at  $2000g$  at  $4^\circ\text{C}$  for 10 minutes and confirmed by a platelet count of  $<10^7/\text{L}$ .

**Assessment of in vivo platelet activation.** Flow cytometric measurements of platelet-monocyte aggregates and platelet surface expression of P-selectin were performed as described previously.<sup>15,16</sup> Immunolabeling was performed in whole blood  $\leq 5$  minutes of collection. Directly conjugated monoclonal antibodies were obtained from DakoCytomation (Cambridge, UK) and Serotec (Oxford, UK). To assess platelet-monocyte aggregates,  $60 \mu\text{L}$  of blood were incubated for 15 minutes with a fluorescein isothiocyanate (FITC)-conjugated anti-CD42a monoclonal antibody (platelet marker) and a

phycoerythrin (PE)-conjugated anti-CD14 monoclonal antibody (monocyte marker) before fixation and erythrocyte lysis with  $500 \mu\text{L}$  of FACSlyse solution (Becton Dickinson, Oxfordshire, United Kingdom).<sup>17</sup>

Cells were measured by flow cytometry (EPICS XL2; Beckman-Coulter). Samples were analysed with EXPO 32 software (Cytometry Systems). Platelet-monocyte aggregates were detected by gating for cells that were positive for both CD14 PE and CD42a FITC. Platelet surface expression of P-selectin was assessed by gating for cells that were positive for both FITC-conjugated anti-CD42a monoclonal antibody, (platelet marker) and PE-conjugated anti-CD62P monoclonal antibody (thrombin receptor-activating peptide [TRAP 1], immunoglobulin G1). Isotype controls were used to reduce error from nonspecific binding.

Platelet microparticles were identified by both size and expression of platelet markers CD41 (glycoprotein [GP] IIb) and CD31 (GPIIIa; platelet endothelial cell adhesion molecule-1). Aliquots ( $25 \mu\text{L}$ ) of platelet-poor plasma were incubated for 30 minutes with a PE-conjugated anti-CD31 monoclonal antibody and a FITC-conjugated anti-CD41 monoclonal antibody (Serotec, Oxford, UK), before dilution with phosphate-buffered saline to form a volume of 1 mL.

Platelet microparticles were gated according to their size (events  $<1.0 \mu\text{m}$ ) by assessment of their forward light scatter. TruCOUNT beads of  $1.0 \mu\text{m}$  (Becton Dickinson) of a known concentration were used to calculate the volume of sample analyzed over 120 seconds at medium flow rate. This allowed the absolute number of platelet microparticles to be measured. Isotype controls were used to reduce error from nonspecific binding. Platelet microparticles were detected by gating for events that were sized  $<1 \mu\text{m}$  (based on forward scatter) and positive for both CD31 and CD41.

**Assessment of in vivo monocyte activation.** Monocyte activation was assessed by flow cytometric measurement of percentage of monocyte CD40 expression and mean fluorescent intensity (MFI) of monocyte CD11b expression, as described previously.<sup>15,16</sup> Immunolabeling was performed in whole blood within 5 minutes of collection. To evaluate CD40 and CD11b on monocytes, blood was diluted 1:2 with phosphate-buffered saline and incubated with the following monoclonal antibodies: anti-CD14:FITC (Serotec), anti-CD40:PE (Serotec), anti-CD11b:PE (Serotec), and appropriate isotype-matched controls for 20 minutes before fixation and erythrocyte lysis with  $500 \mu\text{L}$  of FACSlyse solution. Monocytes were identified by gating for CD14-positive cells.

**Statistical analysis.** Data are shown as scatter plots or mean  $\pm$  standard deviation. Data were analyzed by analysis of variance,  $\chi^2$  and Bonferroni post hoc tests, where appropriate, using Prism 4 software (GraphPad, La Jolla, Calif). Statistical significance was taken as a two-sided value of  $P < .05$ .

**Table II.** Participant demographics

Variable <sup>a</sup>	Healthy volunteers (n = 30) Mean ± SD	Intermittent claudication (n = 30) Mean ± SD	Critical limb ischemia (n = 30) Mean ± SD	Acute coronary syndromes (n = 30) Mean ± SD	Arthroplasty (n = 30) Mean ± SD
Age, y <sup>a</sup>	59 ± 3	60 ± 4	68 ± 2	58 ± 2	57 ± 4
Male sex <sup>b</sup>	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
CV risk factors	16 (53)	22 (73)	23 (77)	20 (67)	17 (57)
Hypertension	0	18 (60)	24 (80)	20 (67)	0
Diabetes	0	7 (23)	10 (33)	8 (27)	0
CAD	0	10 (33)	16 (53)	13 (43)	0
Current smoker	0	17 (57)	16 (53)	16 (53)	0
Medications					
Aspirin	0	30 (100)	30 (100)	30 (100)	0
Clopidogrel	0	0	0	30 (100)	0
Statin therapy	0	30 (100)	30 (100)	24 (80)	0
ACE inhibitor	0	3 (10)	12 (40)	21 (70)	0
β-blocker	0	2 (6.7)	6 (20)	16 (53)	0

ACE, Angiotensin converting enzyme; CAD, coronary artery disease; CV, cardiovascular.

<sup>a</sup>P = .38, one-way analysis of variance.<sup>b</sup>P = .24,  $\chi^2$  analysis.

## RESULTS

Participants were predominantly middle-aged men, with groups having similar distribution of ages and no significant difference in mean age (Table II). In keeping with their clinical presentation, patients with peripheral arterial and coronary heart disease had a range of cardiovascular risk factors and medications that were not present in the healthy volunteers or patients undergoing orthopedic surgery (Table II). Of the 30 patients with CLI, 18 underwent femoral-popliteal bypass and 12 underwent below knee amputations. None underwent endovascular procedures. Dry gangrene was present in 17, but none had wet gangrene.

**Baseline platelet activation.** Platelet activation markers were lowest in healthy volunteers and patients scheduled for arthroplasty (Fig 1). Baseline platelet-monocyte aggregation ( $41.7\% \pm 12.2\%$ ) and platelet microparticles ( $178.7 \pm 106.9$ ) were highest in patients with CLI compared with all other groups (Fig 1). Although patients with CLI had higher values of platelet P-selectin than healthy volunteers or those undergoing arthroplasty ( $P < .001$ ), there was no demonstrable difference between these patients and those with claudication or non-ST elevation MI ( $4.4\% \pm 2.0\%$  vs  $4.2\% \pm 2.0\%$  and  $4.9\% \pm 2.2\%$  respectively;  $P > .05$ ; Fig 1).

**Baseline monocyte activation.** Monocyte activation markers were lowest in healthy volunteers and patients scheduled for arthroplasty (Fig 2). Baseline monocyte expression of CD40 ( $70\% \pm 12.2\%$ ) was highest in patients with CLI compared with all other groups (Fig 2). Baseline monocyte CD11b was greatest in patients with CLI compared with all groups except those with claudication, where it was equivalent ( $56.6 \pm 18.3$  vs  $50.5 \pm 13.9$ , respectively;  $P > .05$ ; Fig 2).

**Perioperative platelet and monocyte activation.** Throughout the perioperative period, levels of all platelet

and monocyte markers remained greater in patients with CLI than in those undergoing arthroplasty (Fig 3 and 4;  $P < .0001$ ). Platelet and monocyte activation rose immediately postoperatively in patients undergoing joint arthroplasty ( $P < .05$ ) before falling on the first postoperative day (Fig 3 and 4). In contrast, platelet activation fell immediately after surgery in patients undergoing infrainguinal revascularization or amputation ( $P < .05$ ; Figure 3), whereas monocyte activation remained unchanged (monocyte CD40,  $P > .05$ ) or rose on day 1 (monocyte CD11b;  $P < .05$ ; Fig 4).

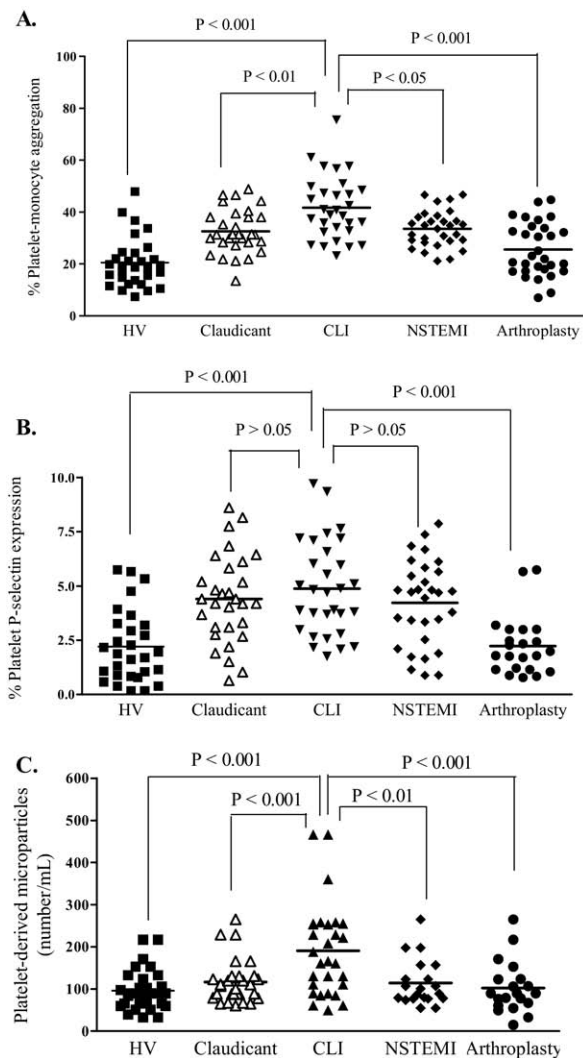
There was no statistically significant difference in markers according to type of surgery performed for CLI (bypass or amputation) and no difference in postoperative trend (subanalysis not shown).

## DISCUSSION

Consistent with previous studies,<sup>19-21</sup> we have demonstrated that platelet and monocyte activation is increased in patients with PAD. We have shown for the first time, to our knowledge, that patients undergoing surgery for CLI have even greater levels of platelet and monocyte activation than patients being treated for acute MI. In addition, perioperative platelet and monocyte activation is markedly increased in these patients and exceeds the increase in platelet activation and inflammation attributable to surgery itself. This study supports the need for an increased appreciation of the cardiovascular risks associated with these patients and an improvement in cardioprotective management, especially in the perioperative period.

PAD affects nearly 30 million people in Western Europe and North America, and up to three-quarters of these patients have coexistent coronary artery disease and a three-fold increased risk of cardiovascular events and death.<sup>25</sup> Despite attempts to raise awareness of PAD as an important marker of cardiovascular risk, patients are often poorly

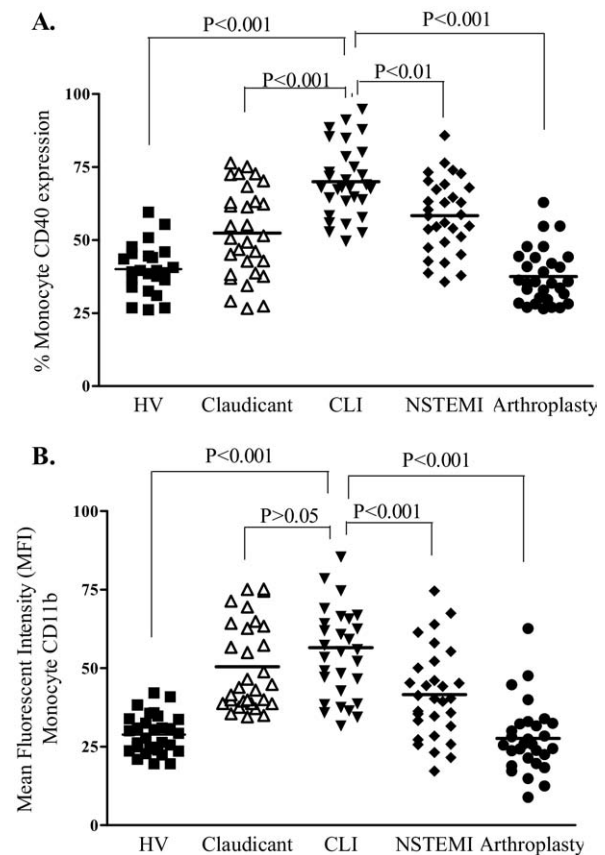




**Fig 1.** Scatter dot plots are shown for resting in vivo platelet activation in healthy volunteers (HV, ■), claudicants (Δ), patients with critical limb ischemia (CLI, ▼), patients with non-ST elevation myocardial infarction (NSTEMI, ◆), and patients undergoing arthroplasty (●). **A**, Percentage of platelet-monocyte aggregation. **B**, Percentage of platelet P-selectin expression. **C**, Platelet-derived microparticles (/mL). The horizontal lines represent population means (n = 30 per group). Analysis by one-way analysis of variance with Bonferroni post-tests.

provided with evidence-based therapies such as antiplatelet and lipid-lowering medications.<sup>26-29</sup> The reasons for this are unclear, but appear to be related to a lack of awareness amongst health professionals of the severity of the disease.

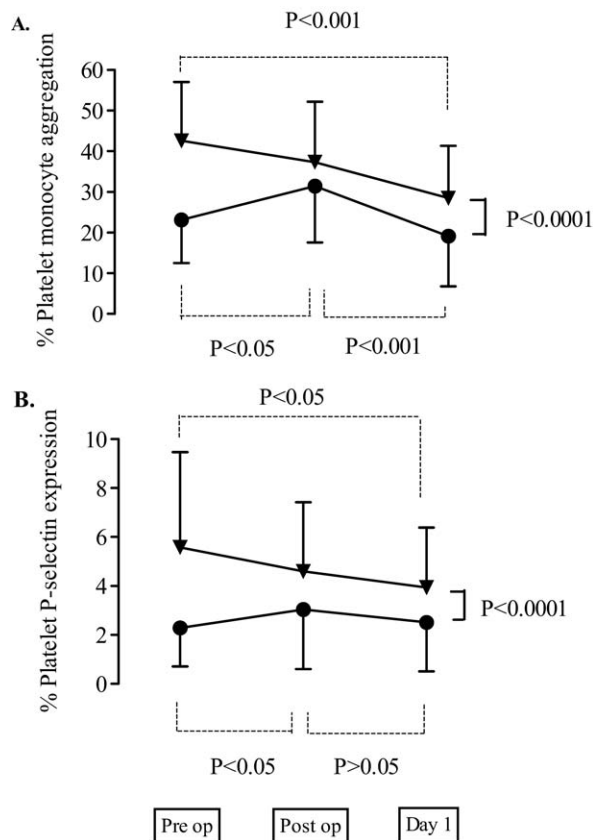
Our study demonstrated that patients with CLI have a greater elevation in systemic markers of platelet and monocyte activation than comparator groups. We acknowledge that the healthy volunteers and patients undergoing arthroplasty did not receive antiplatelet agents or lipid-lowering drugs, which can affect platelet activation. However, patients with PAD have a high incidence of adverse cardio-



**Fig 2.** Resting in vivo monocyte activation in healthy volunteers (HV, ■), claudicants (Δ), patients with critical limb ischemia (CLI, ▼), patients with non-ST elevation myocardial infarction (NSTEMI, ◆), and patients undergoing arthroplasty (●). **A**, Percentage of monocyte CD40 expression. **B**, Monocyte CD11b (mean fluorescent intensity, MFI). Horizontal lines represent population means (n = 30 per group). Analysis by one-way analysis of variance with Bonferroni post-tests.

vascular events despite existing medical therapy. Indeed, the elevations in platelet activation were seen despite the standard medical regimen of aspirin and statin therapy and were even greater than those seen in patients with acute MI. It may be that there is scope for further management of platelet activation in these patients.

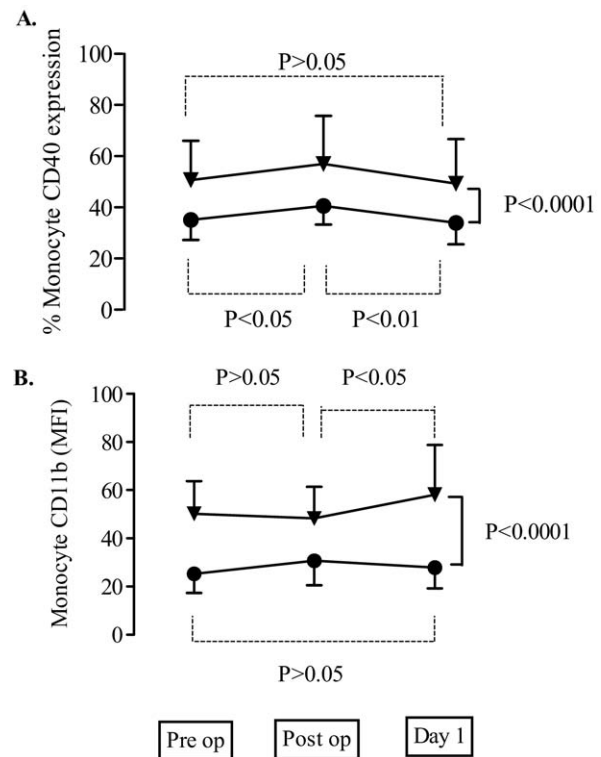
Patients undergoing peripheral vascular surgery have a particularly high incidence of perioperative cardiovascular events. This has been attributed to increased platelet and monocyte activity caused by the surgical process itself. Patients without cardiovascular risk factors undergoing arthroplasty (and therefore no reason for elevated baseline platelet activation) were recruited to assess the effect of surgery alone on platelet activation. Our study showed that throughout the perioperative period, platelet and monocyte activation markers were higher in patients undergoing vascular surgery than in those undergoing arthroplasty. However, although platelet markers rose postoperatively in patients undergoing arthroplasty, we were surprised to see



**Fig 3.** Perioperative in vivo platelet activation is shown in patients undergoing surgery for critical limb ischemia (▼) and arthroplasty (●). Platelet activation in patients undergoing infrainguinal bypass or amputation was greater than in patients undergoing arthroplasty throughout the perioperative period ( $P < .0001$ ). In vivo platelet activation measured by (A) percentage of platelet-monocyte aggregation ( $P < .05$ ) and (B) percentage of P-selectin expression ( $P < .05$ ), rose immediately postoperatively in patients undergoing arthroplasty but fell in those with critical limb ischemia ( $P < .001$ ). Mean  $\pm$  standard deviation; analysis between patient groups by two-way analysis of variance with repeated measures; analysis between time points for the same patient group by one-way analysis of variance with Bonferroni post-tests.

that platelet activation fell immediately after surgery in those with CLI. Patients with PAD, especially CLI, have high baseline levels of activated platelets and monocytes. This is due to the underlying endothelial dysfunction and tissue ischemia. We therefore propose that the lack of response after surgery in these particular patients could be due to the removal of thrombotic stimulus by amputation or revascularization. These patients may benefit from increased platelet inhibition before surgery.

Cardiovascular disease is a critical public health issue. The prevalence of the disease and increased awareness of the cost-benefit associated with the management of cardiovascular risk have led to the concept of potential screening programs for vascular disease.<sup>30</sup> In addition, recommendations are required for the most appropriate use of interven-



**Fig 4.** Perioperative monocyte activation in patients undergoing surgery for critical limb ischemia (▼) and arthroplasty (●). Monocyte activation in patients undergoing infrainguinal bypass or amputation was significantly greater than in patients undergoing arthroplasty throughout the whole perioperative period ( $P < .0001$ ). A, Percentage monocyte CD40 expression tended to rise immediately postoperatively in orthopedic patients before returning to baseline ( $P < .05$ ). B, There was no significant postoperative change in monocyte CD11b (mean fluorescent intensity) in arthroplasty patients ( $P > .05$ ), but levels appeared to rise on day 1 after surgery in those with critical limb ischemia ( $P < .05$ ).

tions and therapies for patients with different manifestations of PAD. The use of imaging technologies or biomarkers could help risk-stratify patients and guide management. This study demonstrates that sensitive markers of in vivo platelet and monocyte activation—known to be predictors of clinical risk—are markedly elevated in patients with severe PAD at the time of surgery, despite current medical therapy. This is in line with reports of the increased cardiovascular risk associated with reducing ABPI.<sup>2</sup>

Further work is required, however, to demonstrate the link between platelet activation and adverse cardiovascular outcomes in this specific patient group. We are therefore conducting a trial of dual antiplatelet therapy (aspirin plus clopidogrel) vs aspirin alone, in patients undergoing surgery for CLI. The effect of intensive antiplatelet therapy on platelet markers, cardiac troponin, and high-selective C-reactive protein will be noted. This may inform the design of larger-scale clinical trials powered at examining clinical end points in this population.

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## AUTHOR CONTRIBUTIONS

Conception and design: AB, AN, SH, DN

Analysis and interpretation: AB, AD, DN

Data collection: AB, NC, AD

Writing the article: AB, AN, OJG, DN

Critical revision of the article: AB, AN, NC, SH, OJG, AD, DN

Final approval of the article: AB, AN, NC, SH, OJG, AD, DN

Statistical analysis: AB

Obtained funding: AB

Overall responsibility: DN

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